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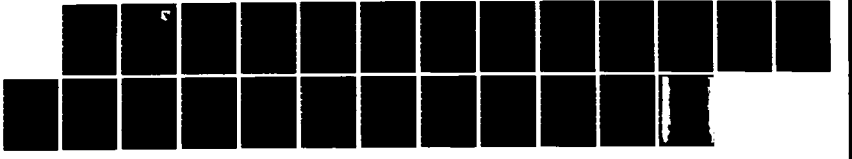

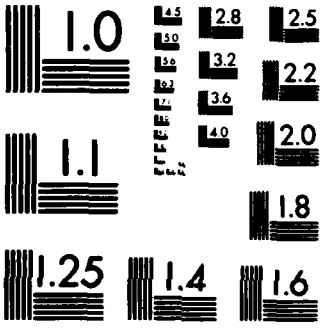

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USE OF SPERM ENZYMES TO DETECT GENOTOXIC AGENTS

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TECHNICAL REVIEW AND APPROVAL

AFAMRL-TR-84-020

The experiments reported herein were conducted according to the "Guide for the Care and Use of Laboratory Animals," Institute of Laboratory Animal Resources, National Research Council.

This report has been reviewed by the Office of Public Affairs (PA) and is releasable to the National Technical Information Service (NTIS). At NTIS, it will be available to the general public, including foreign nations.

This technical report has been reviewed and is approved for publication.

FOR THE COMMANDER

BRUCE O. STUART, PhD
Director Toxic Hazards Division
Air Force Aerospace Medical Research Laboratory

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The sperm enzyme test (SET) system consists of a series of histochemical assays that can be used to assess the functionality of sperm. The objective of this research was to determine the usefulness of the SET system in evaluation of germ cell damage which might be caused by a variety of chemicals. Mice were treated with several known mutagenic or teratogenic agents, or chemicals toxic to the reproductive system two and six weeks before collecting sperm. Sperm was stripped from the vas deferens and examined for count, motility, and the sperm enzymes, acrosin, hyaluronidase, succinic dehydrogenase (SDH), and alpha glycerolphosphate dehydrogenase (aGDH) activities.

Groups of mice were treated through intraperitoneal (i.p.) or inhalation (i.h.) routes. Two weeks after treatment, the chemicals ethylnitrosourea (ENU), ethyl methanesulfonate (EMS), hydroxyurea (HU), cigarette smoke condensate (CSC), dimethyl methylphosphonate (DMMP), perfluoro-n-decanoic acid (PFDA), ethylene dibromide (EDB), and benzene were

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found to be positive by the SET system. At six weeks, only ENU, HU and DMMP exposures caused enzyme changes significantly different from controls, while only ENU and HU caused reduced testis weight, a conventional method of germ cell damage assessment. Two weeks after i.h. treatment, benzene and EDB were found to be positive for sperm enzyme changes, while at six weeks postexposure only DMMP was detected.

Since the sperm which were tested two weeks after treatment were fully formed at the time of treatment, the two week results demonstrate the direct toxicity of these chemicals on sperm enzymes. The six week group sperm are derived from cells treated at preleptotene-late spermatogonial stage. Sperm dysfunction in this group indicates either developmental or mutagenic damage to these germ cells by the active chemical.

The results indicate that the SET system should be included in the routine genotoxic tests for evaluation of reproduction of unknown chemicals in animals and man.

PREFACE

This study was conducted from February 1983 to February 1984, by Western Michigan University, Department of Biomedical Sciences under Air Force Contract No. F33615-83-K-0504. The research was supported by Laboratory Directors Funds. It was performed in support of Project 6302, "Occupational and Environmental Toxic Hazards in Air Force Operations, Task 01, Work Unit 62, "Use of Sperm Enzymes to Detect Genotoxic Agents". Inhalation exposures were performed by personnel from the Air Force Aerospace Medical Research Laboratory's Toxic Hazards Division.

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INTRODUCTION

When chemicals are assessed for safety, the study of their effects on germ cells warrants attention because insults to these cells may result in fertility and/or genetic problems. Sperm, the end product of a long and complex process of spermatogenesis, offers an easy and simple system to study such effects. Until recently, the most common parameters used to detect germ cell damage were sperm motility, count, morphology and fertility index (Balsey et al., 1980; Kluew, 1983). Wyrobek and Bruce (1975) proposed the use of sperm morphology to detect possible genotoxic agents. Over the years this test has been sufficiently validated by screening approximately 182 chemicals covering a wide range, including mutagenic, carcinogenic, and teratogenic agents, and chemicals toxic to the reproductive system (see review by Wyrobek et al., 1983). The shortcomings of this test system, however, lie with the fact that it does not provide adequate information about sperm function, particularly with respect to reproductive potential.

As a supplement to the sperm head shape abnormality test, several investigators have suggested the use of histochemical assays to examine the function of enzymes in single sperm after chemical treatment (Burkhart et al., 1982; Ginsberg et al., 1982; Ficsor et al., 1983). The sperm enzyme tests (SET) have been successful in detecting chemically-induced dysfunction in sperm which had normal head shapes (Ficsor et al., 1984a). However, the variety of chemicals so far examined by the SET system is limited. Also, most studies utilized the convenient intraperitoneal (i.p.) route of exposure which is different from the usual route of exposure encountered in humans.

This study examined the effects on germ cells of several mutagenic, teratogenic, or carcinogenic agents, and chemicals toxic to the reproductive system following i.p. or inhalation (i.h.) routes of exposure using the SET system to measure the enzymes acrosin, hyaluronidase, succinic dehydrogenase (SDH), and alpha glycerolphosphate dehydrogenase (aGDH), as well as the traditional sperm count and motility evaluations.

METHODS

Chemicals: Perfluoro-n-decanoic acid (PFDA), dimethyl methylphosphonate (DMMP), ethylene dibromide (EDB) and benzene were purchased from Aldrich Chemical Company, Milwaukee, WI. Ethyl methanesulfonate (EMS) was from K and K Laboratories, Plainview, NY. N-Ethyl-N-nitrosourea (ENU) was a gift from David Swenson, Upjohn Company, Kalamazoo, MI. Cigarette smoke condensate (CSC) was purchased from Arthur Vaught, University of Kentucky, Lexington, KY. Two grams of the CSC was prepared by smoking 52 low nicotine research cigarettes (2AI) and trapping smoke in a condenser at low temperature.

Animals: 8-10 week old HA (ICR) male mice used were from Harlan Industries (Indianapolis, IN) and were fed Purina Lab Chow ad libitum. Day length was 12h and room temperature was approximately 23°C.

Treatments: For i.p. exposure (Exp 1), mice were treated once by injection with ENU (10, 50, 100 mg/kg), EMS (30, 150, 300 mg/kg), HU (500, 2500, 5000 mg/kg), PFDA (5, 25, 50 mg/kg), DMMP (500, 2500, 5000 mg/kg), or CSC (100, 500, 1000 mg/kg). The solvent used was 0.9% NaCl except for CSC which was suspended in vegetable oil and PFDA which was dissolved in 1:1 propylene glycol/water. Treatment doses were one tenth, one half and maximum tolerated dose (MTD) of each compound. MTD was determined in a pilot experiment (Phase 1). In Exp 1, groups of 10 mice were treated with 10 ml solvent with or without a test agent per kg body weight .

The treatment of mice, 5 to 10 per group, by inhalation (Exp 2) was carried out at the Air Force Aerospace Medical Research Laboratory, Toxic Hazards Division, Wright-Patterson AFB, OH. Mice were exposed to EDB (25 and 100 ppm) and benzene (50 and 500 ppm) for 5 days, 3 hr/day in a 30 L Leach style inhalation chamber (Leach, 1963) at an air flow rate of 5 L/min and 10 L/min, respectively. Similarly, mice were exposed to DMMP (25 and 250 ppm) for 5 days, 24 hr/day in Thomas domes (Thomas, 1966) at an air flow rate of 90 ft^3/min. Mice exposed to air under identical conditions served as controls. The chemical concentrations were monitored by a Hewlett Packard 5880 gas chromatograph and sampling loop.

The animals were killed by cervical dislocation 2 or 6 weeks following treatment. The body weights were recorded before treatment, two or three times weekly and before sacrifice. At weighings, the animals were checked visually for signs of toxicity.

Sperm were stripped from the vas deferens and placed in 1.0 ml of sperm ringer solution (Ficsor et al., 1983). The sperm suspension was divided into 3 parts, washed and subsequently resuspended in an appropriate volume of either phosphate buffered saline (PBS) or sperm ringer as shown in figure 1. The sperm subsamples thus obtained were used for sperm count, motility, acrosin, hyaluronidase, SDH, and aGDH assays.

Sperm count and motility assay: In Exp 1, sperm number and motility were estimated visually on a scale where 1+ represented low sperm count or motility and 4 represented normal sperm number and motility. In Exp 2, approximately 200 sperm per animal were used to determine motility using a Neubauer hemocytometer (Ficsor and Ginsberg, 1980).

Acrosin Assay: Acrosin activity was determined according to methods described by Ficsor et al. (1983). Briefly, sperm were spread on a gelatin-substrate film, and incubated for 30 min at 37°C in a wet box. Slides were then stained with 0.3% toluidine blue and examined. Sperm with gelatin-free halos were scored as having active acrosin.

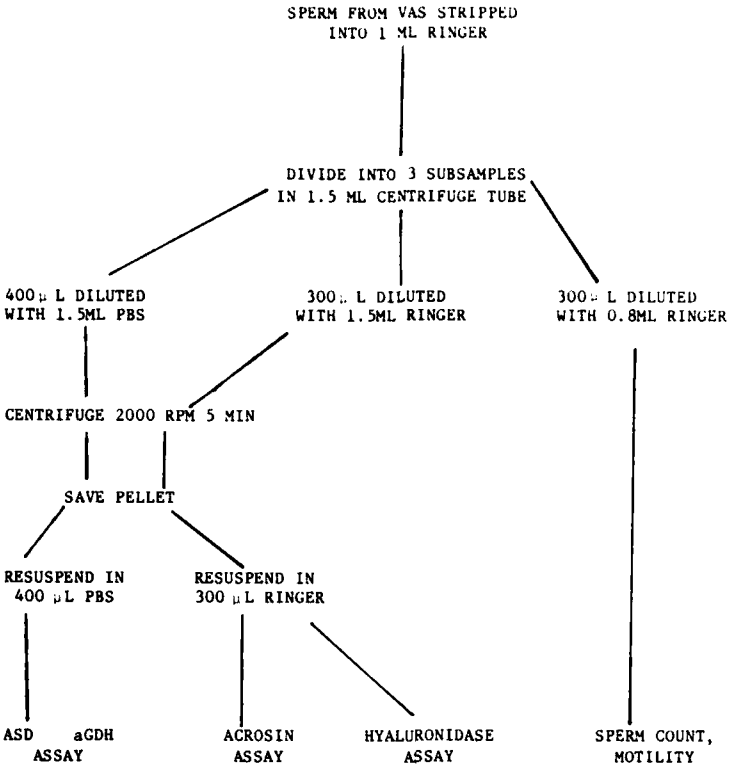

Figure 1. Flow Chart for Sperm Preparation for Sperm Enzyme Tests

Hyaluronidase Assay: Hyaluronic acid-coated Gelbond (FMC corp, Rockland, ME) sheets were cut into 20x50 mm rectangles and mounted on microscope slides (Waibel et al., 1983). Ten 1 of a mouse sperm suspension were spread onto each slide and incubated in a wet box for 3 hr at 37°C. After incubation, slides were fixed for 15 minutes in formaldehyde/acetone (20 ml of 37% formaldehyde mixed with 80 ml acetone) and stained in a solution of 0.3% toluidine blue and 0.2% fast green FCF in 80% acetone/water containing 0.5% acetic acid. The films were destained in a solution of 2% acetic acid in 80% acetone/water. The fixed and stained slides were scored for hyaluronidase activity as demonstrated by dark purple halos around the sperm head.

Succinic Dehydrogenase Assay: A reaction mixture was prepared using 0.75 ml PBS, 1.00 ml 7% sodium succinate, and 1.25 ml 0.3% p-iodonitrotetrazolium violet (INT). One hundred microliters of this mixture were added to each sperm subsample which was then spread on the surface of a slide and immediately incubated for 1 hr at 37°C in a wet box. Following incubation, slides were placed in a horizontal position and dried with warm air from a hair dryer. The slides were washed for 5 minutes in distilled water, dried, again and stored at room temperature. Just prior to examination, the slides

were covered with 15% polyvinyl alcohol in water and a coverslip and scored using a phase contrast microscope at 400x. The quality of the SDH dependent stain decreased when mounted slides were reexamined after overnight storage. Sperm with SDH activity have bright orange midpieces, whereas sperm without activity appear white.

Alpha Glycerolphosphate Dehydrogenase Assay: A reaction mixture was prepared using 7.5 ml Tris HCl Buffer (pH 8.0), 0.13 ml 0.1 M $MgCl_2$, 1.0 ml 0.3% INT, and 25.0 mg alpha glycerolphosphate. One hundred µl of each sperm subsample were spread onto clean premarked slides. The slides were allowed to stand vertically to drain excess liquid and dry. Several drops of reaction mixture were placed on the surface of the slide which was then incubated at 37°C for 25 minutes in a wetbox. Following incubation, as in the case with SDH assay, the slides were dried with warm air, washed for 2 minutes in distilled water, dried, and stored at room temperature. Mounting and scoring of the slides for aGDH activity was done in the same way as for SDH activity.

For determination of sperm enzyme activity, approximately 250 sperm were scored per animal.

Statistical Analysis: Data were collected blindly. The significance of differences between treated and control groups was determined using the Statistical Package for the Social Sciences program's one-way analysis, regression analysis and Mann-Whitney U tests (Nie et al., 1975).

RESULTS

This project was divided into three phases: determination of maximum tolerated dose (Phase 1), exposure to ENU, HU, EMS, CSC, DMMP, and PFDA i.p. (Exp 1), and to benzene, EDB and DMMP i.h. (Exp 2). The means and standard errors for all values are provided in the appendix.

Exp 1 (i.p. exposure): Ethylnitrosourea (ENU) was chosen as positive control. Significant differences were noted between control and ENU treated groups at 6 weeks using the parameters aGDH, SDH, acrosin, sperm count, motility, and testes weights. All parameters also showed dose dependent changes, as determined by regression analysis by dose (Table 1, Figure 2). At two weeks aGDH, acrosin, sperm count, and testes weight were significantly different from controls (Table 1, Figure 2).

EMS caused significant increases in the frequency of sperm without aGDH, SDH, and acrosin activity two weeks after treatment. No significant differences were noted 6 weeks after treatment with EMS. (Table 1, Figure 2).

HU induced a significant decrease (one-way analysis) in testis weight and increases in the frequency of sperm without aGDH and acrosin activity two weeks after treatment. Both of these endpoints showed dose dependency by regression analysis. Regression analysis also showed increased numbers of sperm with decreased SDH activity ($p=.06$). Six weeks after treatment, the frequency of sperm with inactive aGDH and SDH remained above control levels.

TABLE 1

Effect of Selected Chemicals on Sperm Enzyme Activity, Sperm Count, Motility, and Testicular Weight.

2 Weeks

Experiment 1 (i.p. exposure)

AGENT	aGDH	SDH	ACROSIN	HYAL	SC	MO	TW
ENU	AB		A		AB		AB
HU	A	B	AB				AB
EMS	A	A	AB				
CSC	AB	AB			AB		
DMMP							
PFDA		M			A		

Experiment 2 (i.h.exposure)

BENEZENE	B		A				
EDB		M					
DMMP							

6 Weeks

AGENT	aGDH	SDH	ACROSIN	HYAL	SC	MO	TW
ENU	AB	AB	AB		AB	AB	AB
HU	A	A					
EMS							
CSC							
DMMP	AB						
PFDA	A	M					

Experiment 2 (i.h. exposure)

BENZENE							
EDB							
DMMP		AB					

A = Oneway analysis significance at $p \leq 0.05$
B = Regression analysis $p \leq 0.05$
M = Mann-Whitney U test at $p \leq 0.05$

Intraperitoneal injections of CSC resulted in significant increases in sperm without aGDH and SDH activity and significantly lower sperm counts two weeks after treatment when compared to saline controls. Testes weights were lower in the vegetable oil controls. At 6 weeks only the 500 mg/kg dose showed significant increases in sperm without SDH activity. No other parameters were different between CSC and control groups at either 2 or 6 weeks. (Table 1, Figure 2).

Two weeks after treatment with PFDA there were decreased numbers of sperm with acrosin and SDH activity as well as decreased sperm counts. Significant differences from controls were noted in aGDH and SDH activity at 6 weeks (Table 1, Figure 2).

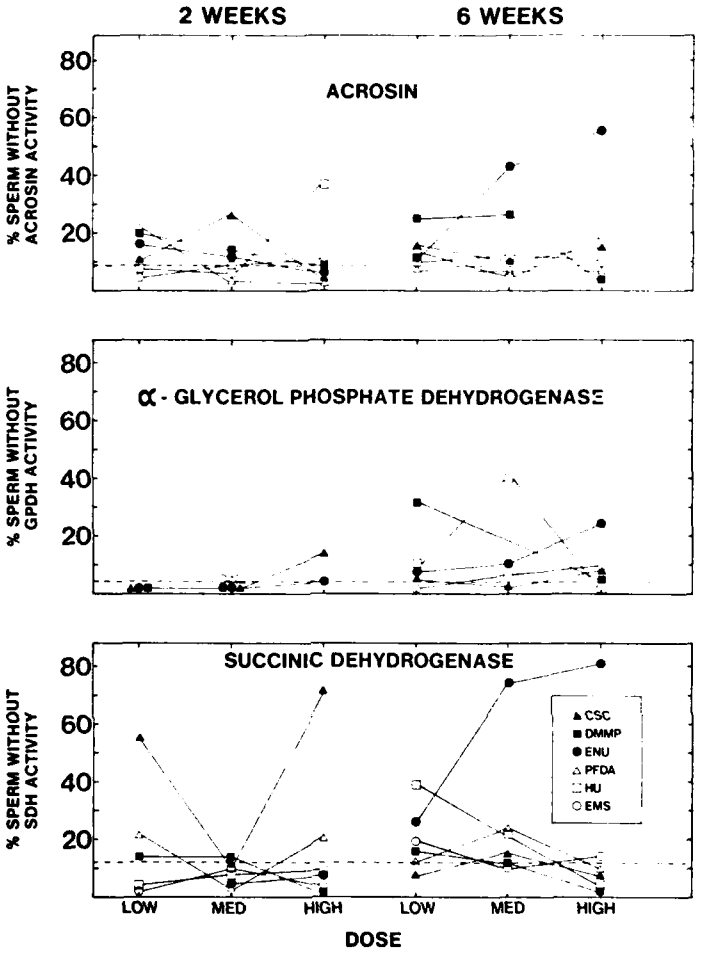

Figure 2. Effect of Selected Chemicals on Sperm Enzyme Activity.

Exp 2 (i.h. exposure): When administered by i.h., DMMP caused a significant increase in the frequency of sperm without SDH activity six weeks after treatment. No effects were noted in the two week group (Table 1, Figure 3). EDB had little effect on the sperm endpoints either at 2 or 6 weeks after i.h. exposure (Table 1, Figure 3).

Benzene showed its only effect 2 weeks after i.h. exposure by significantly altering aGDH activities in sperm. No effects were noted 6 weeks after treatment (Table 1, Figure 3).

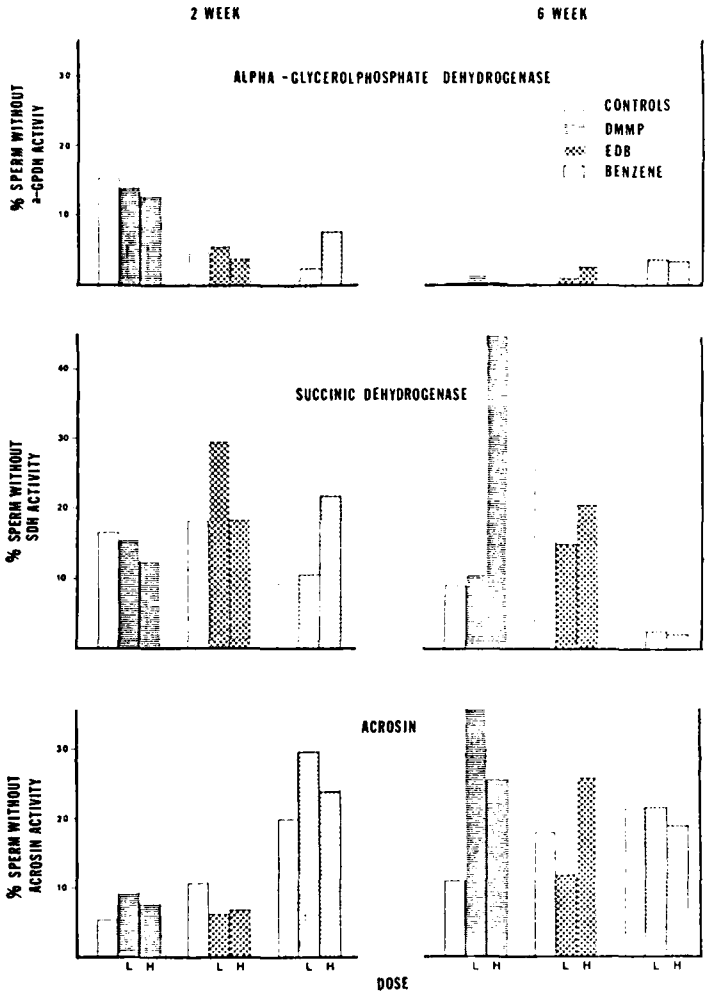

Figure 3. Effect of Inhalation exposure to DMMP, EDB, and Benzene on Sperm Enzyme Activity.

DISCUSSION

Mammalian male germ cells undergo a series of complex development and genetic events which lead to the production of mature sperm capable of fertilization. At any point along this process they are susceptible to damage which may result in their inability to fertilize an ovum. If this damage is induced in preleptotene-late spermatogonial stage (PLSG), evaluated 6 weeks after treatment, the cause may be either genetic or developmental, while damage to spermatozoa (SZ), evaluated 2 weeks after treatment, is likely caused by direct toxicity on the already developed sperm. In any case, a chemical which causes extensive germ cell damage may result in sterility of the male or in genetic damage affecting future generations.

To test both the functional and genetic integrity of sperm, the ideal endpoints would be to see if the sperm successfully fertilized an ovum and produced a "normal" offspring. However, the limitation of such an approach is that, unlike sperm, the ova are few in number and more difficult to obtain. The SET system was thus designed to mimic the functional aspects of the sperm as they approach and penetrate the ovum for fertilization. The acrosomal enzymes acrosin and hyaluronidase are included because they are known to play a key role in the digestion of outer investment of the ovum as the sperm penetrate (McRorie and Williams, 1974), whereas the mitochondrial enzymes SDH and aGDH, located in the midpiece, are included because of their importance in energy metabolism. Enzyme analyses, except for hyaluronidase, have been successfully used to determine germ cell damage from chemical mutagens such as cyclophosphamide, mitomycin C, and ENU (Ginsberg et al., 1981; Ficsor et al., 1983; Ficsor et al., 1984a).

To validate this assay system, representative known teratogens, mutagens, carcinogens, and toxic chemicals were tested. One group of chemicals tested was mutagenic. The positive control for this experiment was ENU, the most potent chemical mutagen known in mice (Russell et al., 1979). As expected, ENU caused significant sperm enzyme abnormalities for all but hyaluronidase at 2 and 6 weeks after treatment. EMS, another known mutagen, acts on post-meiotic cells (Generoso et al., 1974). Bruce and Heddle (1979) have shown that EMS injected once a day for 5 consecutive days induced head shape abnormalities in mouse sperm 5 weeks after the last injection. Our results indicate that a single injection of EMS causes its effects primarily at 2 weeks after injection, supporting its post-meiotic activity. Most parameters returned to control levels by 6 weeks.

A second group of compounds, known carcinogens, are represented in this study by benzene and CSC. Benzene is a known carcinogen and clastogen (Meyne and Legator, 1980) but not teratogenic in rats or mice (Shepard, 1983). In this study, benzene caused direct damage to sperm 2 weeks after treatment, but caused little long term damage as indicated by its reduced effects at 6 weeks. Similarly, CSC had its only effects on sperm enzymes two weeks after treatment. CSC's major active ingredient, benzo(a)pyrene, is both a mutagen and a carcinogen and has caused head shape abnormalities in sperm 5 weeks after i.p. treatment (Bruce and Heddle, 1979). The small effect of CSC on sperm enzymes in this study, however, is in agreement with data obtained in cigarette smoke inhalation studies recently completed. These studies indicated no alterations in sperm enzymes after long-term i.h. exposure to cigarette smoke in mice (Ficsor et al., 1984b).

A third group of compounds are those which are suspected of causing reproductive toxicity. EDB has been shown to be a mutagen in both Neurospora (Malling, 1969) and Salmonella (McCann et al., 1975). EDB is also carcinogenic (Powers et al., 1975). ElJack and Hrudka (1979) showed that 12 days of subcutaneous injections of EDB are toxic to the reproductive system, causing acrosomal and nuclear damage and reduced motility in ram sperm 9 to 12 weeks after the last injection. In none of these studies were experimental animals exposed to EDB by the inhalation route. In contrast to these findings, Haar's review (1980) of existing human epidemiologic data concludes there is no adverse reproductive effects among workers exposed to EDB.

In our study, only one dose of EDB showed significant effects on SDH activity at 2 weeks. The inability to detect alterations in sperm enzymes following inhalation exposure to EDB may be due to the lower level of EDB that reaches the germ cells. A similar explanation may account for the absence of reproductive toxicity in humans exposed to this chemical.

A second reproductive toxicant tested was DMMP. This chemical was administered by both i.p. and i.h. routes. DMMP caused reproductive toxicity in rats after 90 days of administration by gavage. Decreased sperm counts, motility, and fertility as well as dominant lethal effects were observed (Dunnick et al., 1983). These effects correlate well with the data obtained in this study. DMMP caused significant increases in at least one sperm enzyme endpoint at both 2 and 6 weeks after a single i.p. treatment, and at 6 weeks in the i.h. treated groups.

A fourth type of chemical tested is one that is a teratogen but not a mutagen. The chemical chosen was HU. Bruce and Heddle (1979) showed this chemical to be non-mutagenic in both the micronucleus and Salmonella assays but to be positive in causing sperm head shape abnormalities. A number of investigators have shown HU to act as a teratogen, causing CNS, palate and skeletal abnormalities by depressing DNA synthesis (see review by Shepard, 1983). This chemical caused significant differences from controls in several enzymes at both 2 and 6 weeks after treatment. HU's major action occurred at 2 weeks after treatment; by 6 weeks, only the midpiece enzymes were affected.

In a previous experiment (Ficsor and Ginsberg, 1980), a significant reduction in sperm motility was noted 5 weeks after 5 consecutive days of treatment with HU. This effect was not noted in the current study where the single injection regime was used. The single injection may not have inhibited DNA activity long enough to affect the gross motility index but did cause the more sensitive midpiece enzymes, SDH and aGDH, to be affected.

The final chemical type tested was PFDA, a compound known to affect membrane stability (Olson et al., 1983). It caused its major effect at 2 weeks when acrosin, SDH, and sperm count were affected. Only SDH was affected at 6 weeks. The results could be due to direct membrane damage to the developed sperm.

Most of the chemicals detected as positive in the present experiment were mutagenic, teratogenic, or toxic to the reproductive system. For example, ENU is a known teratogen in rats (Shepard, 1983). These multifunctional chemicals undoubtedly caused their effects in PLSG cells by a different mechanism than the one by which they affect already developed SZ (Ficsor et al., 1984a). Therefore, it is important to sample at least two populations of germ cells representing pre-and post-meiotic cells, as was done in the present study. Comparing the efficiency of the sperm enzyme assays for detecting adverse effects clearly shows that, of the midpiece enzymes, aGDH detected more agents than SDH. The only chemical SDH detected but aGDH missed was PFDA. A similar comparison of acrosomal enzymes indicated that acrosin was superior to hyaluronidase. Hyaluronidase was not capable of being altered by any of the chemicals tested. Recent experiments in our laboratory may explain these findings. We have been able to isolate several different hyaluronidases from

sperm, suggesting several potential genes for this enzyme. In addition, data also indicate that hyaluronidase in the vas fluid may bind to sperm, making analysis of hyaluronidase in single sperm very difficult. Until these problems are solved, we do not recommend hyaluronidase assays as a part of the SET system. The combination of aGDH and acrosin assays would have detected effects of every test agent following i.p. exposure. No other combination of any two assays would have been effective. Individually, the aGDH test would have missed both DMMP and PFDA.

In conclusion, the overall results suggest that the sperm enzyme tests may be useful as an addition to current reproduction assessment methods. The assays are not limited to the detection of certain types of damage but detect a wide array of disturbances. Based on these results, we recommend that at least two different germ cell stages be assessed using the enzyme assays for aGDH and acrosin.

LEGEND TO APPENDIX

Significant differences from controls in one-way analysis (a) and Mann-Whitney U test (m) at $p _ 0.05$.

Figures in parenthesis represent number of animals.

PLSG: preleptotene - late spermatogonia, SZ: Spermatozoa

EXPERIMENT	AGENT	ROUTE OF TREATMENT	DOSAGE Mg/Kg	KILLING, WEEKS AFTER TREATMENT	CELL POPULATION TREATED	TESTIS WEIGHT IN Mg ± SE	SPERM NUMBER	SPERM MOTILITY	$ SPERM WITHOUT ●GDH ACTIVITY ± SE.	$ SPERM WITHOUT [illegible] ACTIVITY ± SE.	$ SPERM WITHOUT ACROSIN ACTIVITY ± SE.	$ SPERM WITHOUT HYALURONIDASE ACTIVITY ± SE.
M	SALINE	ip	0	2	SZ	227.78 ± 7.17 [9]	••• [9]	• [9]	0.67 ± 0.65 [9]	8.88 ± 0.65 [9]	7.02 ± 0.43 - [9]	-
			0	6	PLSG	262.00 ± 14.13 [10]	••• [10]	•• [10]	5.59 ± 1.67 [10]	17.77 ± 3.65 [10]	10.8 ± 2.3 [10]	11.[illegible] ± 10.21 [2]
	VEGETABLE OIL	ip	0	2	SZ	128.5 ± 11.60 [10]	-	-	-	10.41 ± 1.21 [10]	11.01 ± 3.31 [9]	5.03 ± 1.14 [6]
			0	6	PLSG	186.0 ± 11.07 [10]	••• [10]	•• [10]	6.01 ± 1.11 [10]	5.90 ± 1.08 [10]	14.01 ± 2.38 [10]	-
	EMU	ip	10	2	SZ	208.00 ± 4.90 [10]	-	-	1.70 ± 1.07	[illegible] ± 1.71[a] [10]	17.33 ± 1.79[a] [10]	-
			50			142.22 ± 14.0[a] [9]	••• [9]	• [9]	0.76 ± 0.15 [9]	6.93 ± 0.28 [9]	12.66 ± 2.21[a] [7]	3.94 [1]
			100			157.78 ± 5.70[a] [9]	••[a] [10]	•• [10]	3.53 ± 0.53 [10]	8.68 ± 1.65 [10]	7.82 ± 0.86 [10]	6.79 ± 2.45 [4]
			10	6	PLSG	257.00 ± 10.86 [10]	••• [10]	•• [10]	13.87 ± 2.61[a] [10]	26.[illegible] ± 3.30 [10]	11.10 ± 2.10 [10]	-
			50			257.70 ± 12.56 [9]	•[a] [9]	•• [9]	14.[illegible] ± [illegible][a] [9]	74.[illegible] ± 1.73[a] [10]	42.[illegible] ± 8.17[a] [7]	24.04 ± 3.02 [2]
			100			198.00 ± 15.[illegible][a] [10]	•[a] [10]	•[a] [10]	23.[illegible] ± [illegible][a] [6]	52.24 ± 1.88[a] [10]	53.21 ± 6.33[a] [5]	8.43 ± 2.81 [7]

EXPERIMENT	AGENT	ROUTE OF TREATMENT	DOSAGE Mg/Kg	KILLING, WEEKS AFTER TREATMENT	CELL POPULATION TREATED	TESTIS WEIGHT IN Mg ± SE	SPERM NUMBER	SPERM MOTILITY	% SPERM WITHOUT NADH ACTIVITY ± SE.	% SPERM WITHOUT SDH ACTIVITY ± SE.	% SPERM WITHOUT ACROSIN ACTIVITY ± SE.	% SPERM WITHOUT HYALURONIDASE ACTIVITY ± SE.
	MU	ip	500	2	SZ	202.0 ± 11.72[a] [10]	-	-	-	2.19 ± 0.25 [10]	8.58 ± 1.72 [7]	-
			2500			178.89 ± 3.81[a] [9]	-	-	3.77 ± 0.43[a] [8]	10.74 ± 2.86 [7]	6.65 ± 1.46 [9]	-
			5000			199.00 ± 9.92[a] [10]	+ [10]	+ [10]	-	9.95 ± 1.60 [10]	33.57 ± 1.45[a] [8]	-
			500	6	PLSG	250.00 ± 15.99 [10]	+++ [10]	++ [10]	16.04 ± 2.15[a] [10]	35.66 ± 2.01[a] [10]	10.32 ± 3.03 [10]	1.88 ± 0.31 [9]
			2500			258.00 ± 8.14 [10]	+++ [10]	++ [10]	40.44 ± 8.16[a] [5]	20.55 ± 0.96 [10]	12.26 ± 2.41 [8]	2.50 ± 1.17 [4]
			5000			266.00 ± 3.10 [10]	+++ [10]	++ [10]	0.96 ± 0.28 [10]	4.44 ± 0.92 [10]	9.70 ± 0.96 [8]	1.75 ± 0.75 [2]
	EMS	ip	30	2	SZ	224.80 ± 12.47 [10]	-	-	-	1.23 ± 0.26 [10]	8.30 ± 1.24 [10]	-
			150			250.9 ± 11.[illegible] [10]	-	-	3.29 ± 1.[illegible] [10]	1.[illegible] ± 1.58[a] [10]	9.31 ± 1.68 [10]	4.99 ± 1.11 [3]
			300			240.[illegible] ± 12.[illegible] [10]	-	-	-	[illegible] ± [illegible] [10]	11.23 ± 2.42[a] [10]	9.50 ± [1]
			30	6	PLSG	253.0 ± 13.58 [10]	+++ [10]	++ [10]	0.65 ± [illegible] [10]	[illegible] ± [illegible] [10]	12.85 ± [illegible] [9]	[illegible] ± [illegible] [7]
			50			288.0 ± 12.14 [10]	+++ [10]	++ [10]	7.95 ± 1.57 [10]	12.[illegible] ± 0.66 [10]	7.64 ± [illegible] [6]	[illegible] ± [illegible] [2]
			300			[illegible].0 ± [illegible] [10]	+++ [10]	++ [10]	6.61 ± 2.19 [10]	15.70 ± [illegible] [10]	17.4 ± 3.62 [illegible]	-

EXPERIMENT	AGENT	ROUTE OF TREATMENT	DOSAGE Mg/Kg	KILLING, WEEKS AFTER TREATMENT	CELL POPULATION TREATED	TESTIS WEIGHT IN Mg ± SE	SPERM NUMBER	SPERM MOTILITY	% SPERM WITH β-GLUCURONIDASE ACTIVITY ± SE	% SPERM WITH ACROSIN ACTIVITY ± SE	% SPERM WITH NO ACROSIN ACTIVITY ± SE	% SPERM WITH NO HYALURONIDASE ACTIVITY ± SE
	CSC	ip	100	2	SZ	231.1 ± 11.36 [9]	++ [9]	++ [9]	0.73 ± 0.2 [9]	56.53 ± 7.54 [9]	10.99 ± 1.73 [8]	-
			500			199.0 ± 5.86 [10]	+++ [10]	++ [10]	0.45 ± 0.25 [10]	9.54 ± 0.88 [10]	25.02 ± 10.94 [8]	-
			1000			224.28 ± 12.88 [7]	++ [7]	++ [7]	16.58 ± 1.86 [9]	71.56 ± 3.65 [9]	4.96 ± 0.89 [7]	-
			100	6	PLSG	263.0 ± 10.3 [10]	+++ [10]	++ [10]	6.78 ± 1.44 [10]	8.39 ± 1.06 [10]	13.67 ± 1.36 [10]	2.98 ± 0.67 [9]
			500			333.5 ± 9.37 [10]	+++ [10]	++ [10]	1.67 ± 0.85 [10]	17.64 ± 1.83 [10]	10.40 ± 1.28 [9]	2.81 ± 0.30 [10]
			1000			278.0 ± 13.15 [10]	+++ [10]	++ [10]	5.17 ± 0.80 [10]	5.42 ± 0.93 [10]	10.92 ± 1.13 [10]	2.67 ± 0.71 [9]
	DMMP	ip	500	2	SZ	233.90 ± 23.58 [10]	-	-	0.63 ± 0.25 [9]	15.04 ± 5.43 [10]	20.63 ± 5.01 [10]	-
			2500			266.0 ± 14.99 [10]	-	-	1.15 ± 0.31 [5]	16.44 ± 5.01 [10]	12.70 ± 2.91 [10]	-
			5000			224.0 ± 11.66 [10]	-	-	-	1.79 ± 0.34 [10]	7.86 ± 1.31 [10]	-
			500	6	PLSG	284.0 ± 15.36 [10]	+++ [10]	++ [10]	34.06 ± 4.44 [9]	16.37 ± 5.02 [10]	24.01 ± 12.73 [7]	-
			2500			296.67 ± 21.67 [9]	+++ [9]	++ [9]	99.07 ± 0.44 [9]	14.30 ± 0.75 [9]	25.04 ± 7.60 [7]	-
			5000			262.5 ± 12.05 [8]	+++ [7]	++ [7]	5.12 ± 0.44 [8]	2.01 ± 0.42 [8]	6.57 ± 1.03 [8]	2.64 ± 0.44 [8]

EXPERIMENT	AGENT	ROUTE OF TREATMENT	DOSAGE MG/KG	KILLING, WEEKS AFTER TREATMENT	CELL POPULATION TREATED	TESTIS WEIGHT IN MG ± SE	SPERM NUMBER	SPERM MOTILITY	% SPERM WITH NO ADH ACTIVITY ± SE	% SPERM WITH NO SDH ACTIVITY/ ± SE	% SPERM WITH ACROSIN ACTIVITY ± SE	% SPERM WITH NO HYALURONIDASE ACTIVITY ± SE
	PFMA	ip	5	2	2S	216.00 ± 0.57 [10]	**[a] [10]	** [10]	0.64 ± 0.67 [7]	.31 ± 10.11 [9]	26.09 ± 3.65[a] [7]	20.00 ± 9.11 [2]
			25			209.5 ± 7.53 [10]	-	-	1.10 ± 0.11 [10]	.63 ± 0.84 [10]	5.35 ± 0.47 [9]	7.10 ± 0.60 [3]
			50			221.00 ± 12.51 [10]	*** [10]	* [10]	-	0.76 ± 2.85[a] [10]	7.30 ± 1.31 [6]	-
			5	5	PLSG	254.00 ± 11.72 [10]	*** [10]	** [10]	6.13 ± 0.9 [10]	11.81 ± 2.85 [10]	10.27 ± 0.91 [7]	4.42 ± 0.90 [10]
			25			275.00 ± 12.22 [10]	*** [10]	** [10]	36.50 ± 1.51[a] [6]	5.33 ± 2.85[a] [10]	9.97 ± 1.91 [7]	1.50 [1]
			50			246.00 ± 8.39 [10]	*** [10]	** [10]	3.45 ± 1.28 [10]	10.11 ± 6.10 [10]	11.87 ± 4.64 [6]	-

EXPERIMENT	AGENT	ROUTE OF TREATMENT	DOSAGE ppm	KILLING WEEKS AFTER TREATMENT	CELL POPULATION TREATED	TESTIS WEIGHT IN g ± SE	SPERM NUMBER 10^6/ml	% Non Motile Sperm	% Sperm without SDH Activity ± SE	% Sperm without ISDH Activity ± SE	% Sperm without Acrosin Activity ± SE	% Sperm without Hyaluronidase Activity ± SE
II	Nitrofurazone	ih 5 hr/day for 5 days at 5 l/min	0	2	SZ	190 (5) 207.5 ± 12.37	2.19 (5) 4.25 ± 1.26	3.87 (4) 7.79 ± 4.68	0.51 ± 0.38 (5) 2.03 ± 1.13	1.13 (5) 19.67 ± 1.85	1.13 (5) 19.95 ± 1.13	
			50			202.33 ± 8.53 (6)	1.78 (6)	4.55 (6)			7.69 (6)	
			500		PLSG	207.50 ± 10.75 (6)	1.55 (6)	2.23 (6)	0.88 ± 0.88 (6)	1.84 (6)	5.62 (6)	
			0	6		201.33 ± 10.55 (6)	1.78 (6)	4.45 (6)	2.03 ± 1.13 (6)	9.87 (6)	7.69 (6)	
			50		SZ	207.5 ± 12.37 (5)	2.19 (5)	3.87 (4)	0.51 ± 0.38 (5)	1.13 (5)	19.95 ± 1.13 (5)	
			0	2		190 (5)	2.19 (5)	3.87 (4)	0.51 ± 0.38 (5)	1.13 (5)	19.95 ± 1.13 (5)	
III	Nitrofurazone	ih 10 l/min for 5 days at 10 l/min	500			195.4 ± 22.87 (10)	1.35 (10)	2.06 (10)	1.08 ± 0.72 (10)	0.55 (10)	2.15 ± 0.68 (10)	
			50			223.0 ± 10.85 (6)	5.12 (6)	2.06 (6)	3.31 ± 1.86 (6)	1.90 (6)	10.85 ± 1.90 (6)	
			500		PLSG	207.50 ± 10.75 (6)	1.55 (6)	2.23 (6)	0.88 ± 0.88 (6)	1.84 (6)	5.62 (6)	
			0	6		201.33 ± 10.55 (6)	1.78 (6)	4.45 (6)	2.03 ± 1.13 (6)	9.87 (6)	7.69 (6)	
			50		SZ	202.33 ± 8.53 (6)	1.78 (6)	4.55 (6)			7.69 (6)	
			0	2		195.4 ± 22.87 (10)	1.35 (10)	2.06 (10)	1.08 ± 0.72 (10)	0.55 (10)	2.15 ± 0.68 (10)	
		ih 5 hr/day for 5 days at 5 l/min	100			262.33 ± 8.06 (6)	4.71 (6)	2.83 (6)	2.05 ± 1.67 (6)	1.22 (6)	22.47 ± 4.00 (6)	
			250			259.00 ± 8.87 (7)	5.83 (7)	1.85 (7)	1.22 ± 0.88 (7)	0.88 (7)	9.05 ± 1.00 (7)	
			0	6	PLSG	274.86 ± 14.01 (7)	2.47 (7)	1.82 (7)	0.0 ± 0.0 (7)	0.0 (7)	25.6 ± 9.57 (7)	
			100			266.71 ± 17.30 (6)	3.57 (6)	3.9 (6)	0.07 ± 0.07 (6)	0.07 (6)	14.87 ± 2.45 (6)	
			250			243.83 ± 16.73 (7)	5.83 (7)	1.85 (7)	0.12 ± 0.08 (7)	0.12 (7)	9.05 ± 1.00 (7)	
			0	6	SZ	271.40 ± 2.27 (5)	2.89 (5)	2.39 (5)	0.2 ± 0.2 (5)	0.2 (5)	44.53 ± 10.92[a] (5)	
			25			259.00 ± 8.87 (7)	5.83 (7)	1.85 (7)	1.22 ± 2.86 (7)	0.88 (7)	9.05 ± 1.00 (7)	
			250			243.83 ± 16.73 (7)	5.83 (7)	1.85 (7)	1.22 ± 2.86 (7)	0.88 (7)	9.05 ± 1.00 (7)	
			0	6	PLSG	274.86 ± 14.01 (7)	2.47 (7)	1.82 (7)	0.0 ± 0.0 (7)	0.0 (7)	25.6 ± 9.57 (7)	
			25			266.71 ± 17.30 (6)	3.57 (6)	3.9 (6)	0.07 ± 0.07 (6)	0.07 (6)	14.87 ± 2.45 (6)	
			100			262.33 ± 8.06 (6)	4.71 (6)	2.83 (6)	2.05 ± 1.67 (6)	1.22 (6)	22.47 ± 4.00 (6)	
		ih 5 hr/day for 5 days at 5 l/min	0	2	SZ	190 (5) 207.5 ± 12.37 (5)	2.19 (5) 4.25 ± 1.26 (5)	3.87 (4) 7.79 ± 4.68 (4)	0.51 ± 0.38 (5) 2.03 ± 1.13 (5)	1.13 (5) 19.67 ± 1.85 (5)	1.13 (5) 19.95 ± 1.13 (5)	
			50			202.33 ± 8.53 (6)	1.78 (6)	4.55 (6)			7.69 (6)	
			500		PLSG	207.50 ± 10.75 (6)	1.55 (6)	2.23 (6)	0.88 ± 0.88 (6)	1.84 (6)	5.62 (6)	
			0	6		201.33 ± 10.55 (6)	1.78 (6)	4.45 (6)	2.03 ± 1.13 (6)	9.87 (6)	7.69 (6)	
			50		SZ	202.33 ± 8.53 (6)	1.78 (6)	4.55 (6)			7.69 (6)	
			0	2		195.4 ± 22.87 (10)	1.35 (10)	2.06 (10)	1.08 ± 0.72 (10)	0.55 (10)	2.15 ± 0.68 (10)	
		ih 10 l/min for 5 days at 10 l/min	500			195.4 ± 22.87 (10)	1.35 (10)	2.06 (10)	1.08 ± 0.72 (10)	0.55 (10)	2.15 ± 0.68 (10)	
			50			223.0 ± 10.85 (6)	5.12 (6)	2.06 (6)	3.31 ± 1.86 (6)	1.90 (6)	10.85 ± 1.90 (6)	
			500		PLSG	207.50 ± 10.75 (6)	1.55 (6)	2.23 (6)	0.88 ± 0.88 (6)	1.84 (6)	5.62 (6)	
			0	6		201.33 ± 10.55 (6)	1.78 (6)	4.45 (6)	2.03 ± 1.13 (6)	9.87 (6)	7.69 (6)	
			50		SZ	202.33 ± 8.53 (6)	1.78 (6)	4.55 (6)			7.69 (6)	
			0	2		195.4 ± 22.87 (10)	1.35 (10)	2.06 (10)	1.08 ± 0.72 (10)	0.55 (10)	2.15 ± 0.68 (10)	

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